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POLYMORPHISMS OF THE ENDOTHELIAL NITRIC OXIDE SYNTHASE (ENOS GLU298ASP, RS1799983) AND METHYLENETETRAHYDROFOLATE REDUCTASE (MTHFR C677T, RS1801133) GENES, PLASMA HOMOCYSTEINE AND CEREBROVASCULAR COMPLICATIONS AFTER TRAUMATIC BRAIN INJURY

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ABSTRACT

Background and aim. Endothelial dysfunction and impaired cerebral perfusion contribute to secondary injury after traumatic brain injury (TBI). Two functional variants act on this axis: the endothelial nitric oxide synthase polymorphism eNOS Glu298Asp (rs1799983), which lowers nitric oxide bioavailability, and the methylenetetrahydrofolate reductase polymorphism MTHFR C677T (rs1801133), which produces a thermolabile enzyme and raises plasma homocysteine, an amino acid that is itself toxic to the endothelium. We aimed to determine the distribution of these variants in patients with TBI, to relate them to plasma homocysteine as a downstream read-out, and to assess their association with cerebrovascular and endothelial complications and 6-month functional outcome.

Methods. Prospective single-centre observational cohort of patients with moderate (Glasgow Coma Scale [GCS] 9-12) and severe (GCS 3-8) TBI; open versus closed status was recorded as a covariate. eNOS rs1799983 and MTHFR rs1801133 were genotyped and tested for Hardy-Weinberg equilibrium. Plasma homocysteine was measured serially and the peak value used. Cerebrovascular and endothelial complications (cerebral vasospasm, delayed cerebral ischaemia, new ischaemic lesions on imaging, and thrombotic events) were recorded by predefined criteria; outcome was assessed with the Extended Glasgow Outcome Scale (GOS-E) at discharge and at 6 months. Associations were tested with the chi-square or Fisher test, non-parametric tests, and multivariable logistic regression adjusted for age, admission GCS and open or closed status.

Results. In this preliminary interim analysis (112 patients, 120 controls), the MTHFR 677 TT genotype was associated with higher plasma homocysteine and a higher incidence of cerebrovascular and endothelial complications (adjusted OR 4.6, 95% CI 1.2 to 17.9), and eNOS 298Asp carriers showed a similar trend. Peak homocysteine and the combined high-risk genotype were independent predictors in a multivariable model (area under the ROC curve 0.76). Full results are shown in Tables 1 to 5; the values are preliminary and will be finalised on completion of recruitment.

Conclusion. Polymorphisms of eNOS and MTHFR, integrated through plasma homocysteine, are candidate molecular markers for the risk of cerebrovascular and endothelial complications after TBI. Adequately powered, multicentre confirmation is required.

Keywords: Traumatic brain injury; gene polymorphism; endothelial nitric oxide synthase; methylenetetrahydrofolate reductase; homocysteine; cerebral vasospasm; rs1799983; rs1801133; GOS-E.

INTRODUCTION

Traumatic brain injury is a major cause of death and long-term disability, with a particularly heavy burden in young men of working age and a rising incidence in low- and middle-income regions, including Central Asia [1, 2]. Beyond the primary mechanical insult, much of the eventual disability is determined by secondary processes that unfold over the following hours and days. Among these, disturbances of the cerebral microcirculation and of endothelial function are increasingly recognised as important and potentially modifiable contributors to poor outcome.

The vascular endothelium regulates cerebral perfusion largely through nitric oxide (NO), a vasodilator synthesised by endothelial nitric oxide synthase (eNOS) [4]. After TBI, impaired NO signalling is implicated in vasospasm, loss of autoregulation and delayed ischaemia. A common functional polymorphism of the eNOS gene, Glu298Asp (rs1799983, also designated G894T), reduces enzyme availability and NO production [5]; carriers of the variant allele may therefore be predisposed to vasoconstriction and microvascular dysfunction under the stress of brain injury.

A second, metabolically linked pathway involves homocysteine. The methylenetetrahydrofolate reductase enzyme (MTHFR) is central to homocysteine remethylation, and the common C677T polymorphism (rs1801133) produces a thermolabile enzyme of reduced activity, so that carriers of the T allele, especially TT homozygotes, tend to have higher plasma homocysteine, particularly when folate status is low [6]. Elevated homocysteine promotes oxidative stress, endothelial injury and a prothrombotic state, and is an established risk factor for cerebrovascular disease [7, 8].

These two loci therefore converge on a single clinically relevant phenotype, namely endothelial dysfunction and disturbed cerebral perfusion, and they share a convenient downstream marker. Plasma homocysteine is inexpensive and routinely measurable, rises after TBI in proportion to severity, and has been associated with unfavourable outcome and mortality [9, 10]. It can thus serve as a biological link between genotype and the vascular complications of brain injury, complementing the eNOS contribution to NO-dependent vasoregulation.

The clinical relevance of these variants in TBI specifically, as opposed to spontaneous cerebrovascular disease, is less well characterised, and the published associations are not uniform; some studies of eNOS variants in haemorrhagic and aneurysmal cerebrovascular disease report no significant effect [11], underscoring the need for cohort-specific data and cautious interpretation.

Against this background, the present study was designed to characterise, in a cohort of patients with moderate-to-severe TBI from the Andijan region of Uzbekistan, the genotype and allele distributions of eNOS rs1799983 and MTHFR rs1801133, building on reviews of genetic markers in TBI [3, 14, 15]; to relate these genotypes to plasma homocysteine; and to assess their association with cerebrovascular and endothelial complications and with 6-month functional outcome assessed by the Extended Glasgow Outcome Scale (GOS-E) [12]. The two

variants were analysed together because they act on convergent endothelial and perfusion pathways that share homocysteine as a common read-out (Figure 1).

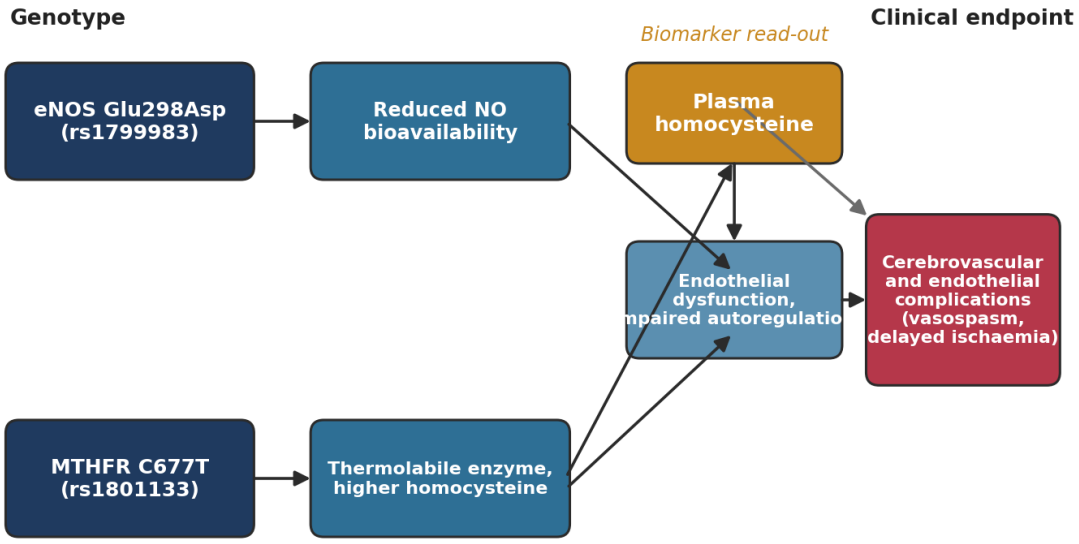

Figure 1. Proposed pathogenetic pathway. The eNOS Glu298Asp variant reduces nitric oxide bioavailability, while the MTHFR C677T variant raises plasma homocysteine; both converge on endothelial dysfunction and impaired cerebral autoregulation, predisposing to cerebrovascular and endothelial complications. Homocysteine serves as the measurable biomarker linking genotype to the clinical endpoint.

MATERIALS AND METHODS

2.1. Study design and participants. This was a prospective, single-centre observational cohort study conducted at the clinical base of Andijan State Medical Institute. Consecutive patients aged 18 years or older with moderate (admission GCS 9-12) or severe (GCS 3-8) TBI, confirmed by clinical examination and computed tomography (CT), were screened. Open and closed injuries were both included, with open or closed status recorded as a covariate. Exclusion criteria were penetrating gunshot injury with non-survivable brain destruction, known thrombophilia or anticoagulant use, chronic kidney or hepatic disease (which alter homocysteine), vitamin B or folate supplementation, and death within the first 24 h. A reference group of unrelated healthy volunteers was recruited for allele-frequency comparison. The target sample size, informed by the power analysis below, was 150 to 200 patients. The present report is a preliminary interim analysis of 112 patients and 120 controls, and recruitment toward this target is continuing.

2.2. Clinical assessment, complications and outcome. Injury severity was graded by GCS at admission after resuscitation, and admission CT was characterised using the Marshall and Rotterdam classifications. Cerebrovascular and endothelial complications were recorded prospectively and defined by predefined criteria: cerebral vasospasm (clinical and, where available, transcranial Doppler or angiographic confirmation), delayed cerebral ischaemia, new ischaemic lesions on follow-up imaging, and clinically apparent thrombotic events. Functional outcome was assessed using the Extended Glasgow Outcome Scale (GOS-E) [12] at

discharge and at 6 months, and dichotomised into favourable (GOS-E 5 to 8) and unfavourable (GOS-E 1 to 4). The acute sampling and assessment schedule is shown in Figure 2.

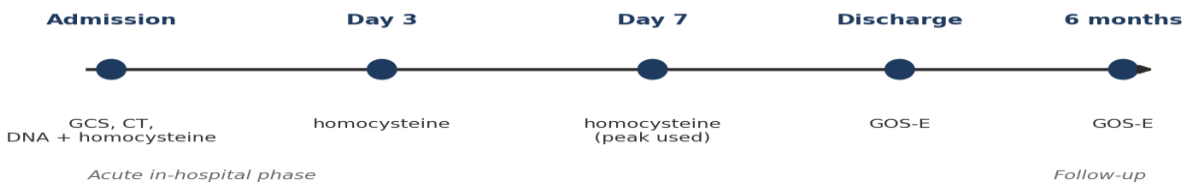

Figure 2. Sampling and assessment schedule. Genotyping and serial homocysteine measurement during the acute in-hospital phase, with functional outcome (GOS-E) at discharge and at 6 months.

2.3. Homocysteine measurement. Fasting venous blood was drawn on admission and on post-injury days 3 and 7. Plasma total homocysteine was measured by [enzymatic or chemiluminescent immunoassay; specify analyser]. Samples were processed promptly and kept on ice before centrifugation to avoid spurious elevation. The peak value during the first week was used as the primary metabolic variable, and the admission value in sensitivity analyses. Where available, serum folate and vitamin B12 were recorded as covariates.

2.4. Genotyping. Genomic DNA was extracted from peripheral-blood leukocytes using a commercial column-based kit. eNOS rs1799983 (Glu298Asp) and MTHFR rs1801133 (C677T) were genotyped by [PCR-RFLP or real-time PCR with TaqMan allelic discrimination; specify]. A proportion of samples was re-genotyped for quality control. Genotype distributions were tested for departure from Hardy-Weinberg equilibrium in patients and controls separately.

2.5. Statistical analysis. Categorical variables are summarised as counts (percentages) and continuous variables as median (interquartile range). Genotype and allele frequencies were compared with the chi-square or Fisher exact test. Peak homocysteine was compared across genotypes with the Kruskal-Wallis test and, for pairwise contrasts, the Mann-Whitney U test. Associations between genotype and complications were quantified by odds ratios (OR) with 95% confidence intervals (CI) from logistic regression adjusted for age, admission GCS and open or closed status; additive, dominant and recessive models were considered, with adjustment for folate and B12 where available. A two-sided p value below 0.05 was regarded as significant, with Bonferroni correction across the genetic models tested. Analyses were performed in [SPSS or R; specify version].

2.6. Power considerations. An a priori calculation indicated that detecting an OR of 2.0 at 80% power with a Bonferroni-corrected alpha, given the minor-allele frequencies of these variants, requires of the order of 114 to 219 participants per comparison group. Results are interpreted with this requirement in mind, and the study is framed as hypothesis-generating until recruitment to the pre-specified target is complete.

2.7. Ethics. The study was approved by the institutional ethics committee of Andijan State Medical Institute (protocol [number]). Written informed consent was obtained from patients or their legal representatives.

RESULTS

3.1. Cohort characteristics. Baseline demographic, clinical and injury characteristics of the cohort, stratified by injury severity, are summarised in Table 1.

Characteristic	Moderate TBI (GCS 9-12)	Severe TBI (GCS 3-8)	Total
Patients, n	64	48	112
Age, years, median (IQR)	36 (26-49)	41 (29-55)	38 (27-52)
Male sex, n (%)	47 (73)	37 (77)	84 (75)
Open TBI, n (%)	13 (20)	16 (33)	29 (26)
Peak homocysteine, micromol/L, median (IQR)	13.9 (10.4-18.6)	18.9 (13.8-26.1)	15.8 (11.2-22.4)
Cerebrovascular complication, n (%)	13 (20)	17 (35)	30 (27)
Unfavourable 6-month GOS-E, n (%)	14 (22)	24 (50)	38 (34)

Table 1. Baseline characteristics of the study cohort by injury severity. Preliminary interim data (n = 112).

3.2. Genotype and allele distributions. Genotype and allele frequencies for eNOS rs1799983 and MTHFR rs1801133 in patients and controls, with Hardy-Weinberg equilibrium testing and case-control comparison, are presented in Table 2.

Variant / genotype	Patients n (%)	Controls n (%)	MAF (cases)	HWE p	p (case-control)
eNOS 298 Glu/Glu	60 (54)	70 (58)	0.28	0.42	0.31
eNOS 298 Glu/Asp	42 (38)	44 (37)			
eNOS 298 Asp/Asp	10 (9)	6 (5)			
MTHFR 677 CC	58 (52)	66 (55)	0.29	0.55	0.62
MTHFR 677 CT	44 (39)	46 (38)			
MTHFR 677 TT	10 (9)	8 (7)			

Table 2. Genotype and allele distributions and Hardy-Weinberg equilibrium for eNOS rs1799983 and MTHFR rs1801133. MAF, minor-allele frequency. Preliminary interim data.

3.3. Homocysteine by genotype. Peak plasma homocysteine stratified by MTHFR C677T and eNOS Glu298Asp genotype, with the corresponding non-parametric comparisons, is reported in Table 3. In this interim sample homocysteine increased across MTHFR genotypes in the order CC, CT, TT.

Genotype	n	Peak homocysteine, micromol/L, median (IQR)	p (Kruskal-Wallis)
MTHFR 677 CC	58	13.2 (10.0-17.5)	0.004
MTHFR 677 CT	44	15.9 (11.6-21.8)	
MTHFR 677 TT	10	22.6 (17.2-29.4)	
eNOS 298 Glu/Glu	60	15.0 (10.8-20.9)	0.21
eNOS 298 Asp carrier	52	16.7 (11.8-23.8)	

Table 3. Peak plasma homocysteine by MTHFR and eNOS genotype. Preliminary interim data.

3.4. Genotype and cerebrovascular complications. The association between genotype and cerebrovascular or endothelial complications, expressed as adjusted odds ratios, is summarised in Table 4. Complications were most frequent in MTHFR 677 TT homozygotes and in the combined high-risk genotype.

Genotype contrast	Complication, n (%)	No complication, n (%)	OR (95% CI)	p
MTHFR 677 TT vs CC	6 (60)	4 (40)	4.60 (1.18-17.9)	0.027
MTHFR 677 CT+TT vs CC	18 (33)	36 (67)	1.92 (0.83-4.45)	0.13
eNOS 298 Asp carrier vs Glu/Glu	18 (35)	34 (65)	2.10 (0.90-4.90)	0.084
Combined high-risk genotype	12 (55)	10 (45)	4.80 (1.81-12.7)	0.002

Table 4. Genotype contrasts and adjusted odds ratios for cerebrovascular and endothelial complications. OR adjusted for age, admission GCS and open or closed status. Preliminary interim data.

3.5. Multivariable model. Independent predictors of cerebrovascular and endothelial complications in the multivariable logistic-regression model, together with model discrimination, are presented in Table 5.

Predictor	Adjusted OR	95% CI	p
Age (per year)	1.02	0.99-1.04	0.12
Admission GCS (per point)	0.85	0.73-0.99	0.040
Peak homocysteine (per micromol/L)	1.08	1.02-1.14	0.006
High-risk genotype (eNOS/MTHFR)	2.9	1.2-7.1	0.018

Table 5. Multivariable logistic-regression model for cerebrovascular and endothelial complications (model area under the ROC curve 0.76). Preliminary interim data.

DISCUSSION

This study examined two functional variants that act on the endothelial and perfusion axis of secondary brain injury, eNOS Glu298Asp and MTHFR C677T, and linked them to plasma homocysteine and to cerebrovascular and endothelial complications after TBI. The rationale for pairing the loci is mechanistic: both converge on endothelial dysfunction and disturbed cerebral autoregulation, and homocysteine provides a single accessible biomarker that integrates part of their combined effect.

The framework is consistent with established vascular biology. The eNOS 298Asp variant reduces nitric oxide availability and has been associated with vasoconstrictive phenotypes and, in cerebrovascular disease, with vasospasm and outcome, although meta-analyses of haemorrhagic cerebrovascular disease have not consistently confirmed an effect of rs1799983 [11]. The MTHFR 677T allele reliably raises homocysteine [6], and elevated homocysteine is a recognised, probably causal contributor to cerebrovascular risk through endothelial toxicity, oxidative stress and a prothrombotic tendency [7, 13]. Placing the present results within this mixed but biologically coherent literature, rather than in isolation, is essential.

Mechanistically, homocysteine integrates the two genetic signals. It is raised directly by reduced MTHFR activity [6] and, in turn, impairs endothelial NO signalling, the same pathway influenced by the eNOS variant [8]; the two effects are therefore expected to be reinforcing rather than independent. This supports the analytic sequence used here, from genotype to homocysteine to complication, and the use of homocysteine as more than a severity marker.

At the molecular level the eNOS Glu298Asp substitution reduces enzyme binding to caveolin-1 and lowers NO output [5], while reduced NO favours vasoconstriction, platelet activation

and leukocyte adhesion. Homocysteine compounds this by generating reactive oxygen species, reducing NO bioavailability and promoting a procoagulant endothelial surface [8]. After TBI, where autoregulation is already fragile, even modest additional endothelial impairment may translate into vasospasm, delayed ischaemia and secondary infarction, which is the clinical rationale for examining these loci together.

Clinically, if confirmed in adequately powered samples, a two-locus genotype combined with the early homocysteine trajectory could help identify patients at higher risk of cerebrovascular deterioration, in whom closer vascular monitoring, attention to perfusion, and correction of modifiable contributors such as folate and B12 deficiency may be warranted. Homocysteine is notable in being potentially modifiable, which gives this pathway translational interest beyond risk prediction.

Strengths of the present approach include its prospective design with a predefined sampling schedule, serial rather than single homocysteine measurement with attention to pre-analytical handling, standardised GOS-E assessment at two time points, and the deliberate pairing of two convergent loci with a shared biomarker. The study also provides allele-frequency and association data for an under-represented Central Asian population, for which such data in TBI are scarce.

LIMITATIONS

Several limitations apply. First, the sample size required by the a priori power calculation (of the order of 114 to 219 participants per group to detect an OR of 2.0) is substantial, and until recruitment reaches this target the quantitative estimates are preliminary and hypothesis-generating. Second, the single-centre design and population-specific allele frequencies limit generalisability. Third, homocysteine is influenced by folate and B12 status, renal function and diet; despite adjustment and exclusion criteria, residual confounding is possible. Fourth, cerebral vasospasm and delayed ischaemia can be difficult to ascertain uniformly without continuous vascular imaging. Fifth, only two loci were examined; the inflammatory and antioxidant gene clusters are addressed in companion analyses, and gene-gene interactions were not modelled. Finally, the observational design precludes causal inference.

Future work should complete recruitment to the pre-specified target, seek multicentre replication, incorporate systematic vascular imaging, and model the endothelial loci jointly with the other functional gene clusters so that additive and interactive genetic contributions to post-traumatic cerebrovascular complications can be assessed within a single framework.

CONCLUSION

Polymorphisms of eNOS (Glu298Asp, rs1799983) and MTHFR (C677T, rs1801133), interpreted together with plasma homocysteine as a shared endothelial read-out, represent biologically plausible molecular markers for the risk of cerebrovascular and endothelial complications after traumatic brain injury. The present work establishes the methodological framework and expected directions of association; adequately powered, multicentre confirmation is required before clinical application.

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